Gluconeogenesis in the amphibian retina

Lactate is preferred to glutamate as the gluconeogenic precursor

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The capacity for gluconeogenesis in the isolated amphibian retina was found to be approx. 70-fold greater with lactate than with glutamate as the gluconeogenic precursor, 1426 versus 21 pmol of glucose incorporated into glycogen/h per mg of protein. It was also found that 11–15% of the glucosyl units in glycogen are derived from C_3 metabolites of the glycolytic pathway, suggesting that lactate is recycled within the retina. In concert with these metabolic observations, a full complement of the gluconeogenic enzymes was detected in retinal homogenates. These included: glucose-6-phosphatase, fructose-1,6-bisphosphatase, acetyl-CoA-dependent pyruvate carboxylase and phosphoenolpyruvate carboxykinase. Agents that regulate the rate of gluconeogenesis in hepatic tissue were tested on the retina. At concentrations of glutamate and lactate that are presumed to be relevant physiologically, it was found that vasoactive intestinal peptide, ionophore A23187 and elevated $[K^+]$ each enhanced the rate of gluconeogenesis in Ringer containing 50 μ M-glutamate, whereas in Ringer containing 8.5 mM-lactate these agents inhibited the rate of gluconeogenesis. Further, it was found that the classic gluconeogenic hormone glucagon inhibited gluconeogenesis in both glutamate- and lactate-containing Ringer. Retinal energy metabolism was found to be altered in lactate-containing Ringer, in that lactate production was suppressed completely. In addition, glycogen metabolism appeared to be dependent on increased cytosolic Ca^{2+} and was insensitive to increased retinal cyclic AMP.

INTRODUCTION

Gluconeogenesis has been defined by Krebs (1963) as 'the formation of carbohydrate from any non-carbohydrate precursors'. It is a general belief that nervous tissue either does not have a gluconeogenic pathway or its capacity for gluconeogenesis is extremely low (Scrutton & Utter, 1968; Lehninger, 1977; Hawkins & Mans, 1983). We reported that the amphibian retina possesses a gluconeogenic pathway (Goldman & Witkovsky, 1987). It was shown that glutamate may be converted back into carbohydrate and the glucose formed stored in glycogen. This conversion is analogous to what is believed to occur in mammalian liver and kidney. In this case lactate and amino acids produced from other tissues are converted back into glucose in order to maintain glucose homeostasis (Lehninger, 1977). By analogy, it was suggested that the gluconeogenic pathway in retina may be important in the recycling of glutamate; the glucose derived metabolically from glutamate could be used as an energy source by other retinal neurons (Goldman & Witkovsky, 1987).

The purpose of the present study is to evaluate the importance of the gluconeogenic pathway in the retina. In this report it is shown that the gluconeogenic pathway in the amphibian retina may maintain glucose homeostasis within the tissue, and further, that the activity of the pathway is far greater with lactate as the precursor for glucose, suggesting that lactate is recycled. Finally it is shown that, in the presence of lactate, retinal

metabolism is altered, in that its well-known capacity for aerobic glycolysis is abolished.

EXPERIMENTAL

Preparation and incubation of the tissue

Bullfrogs (*Rana catesbeiana*) obtained from commercial vendors were anaesthetized with 3-aminobenzoic acid ethyl ester (MS 222). After the retinas were removed with adhering pigment epithelium and transferred to oxygenated medium, the frogs were decapitated and pithed. The composition of the amphibian Ringer was (mM): NaCl, 100; KCl, 2.5; $NaHCO_3$, 20; KH_2PO_4 , 1.0; $MgCl_2$, 1.2; $CaCl_2$, 1.8; glucose, 10. The concentrations of the other substances used are given in the Tables. The solution was gassed previously with O_2/CO_2 (19:1) to pH 7.4. The pigment epithelium was peeled carefully from each of the retinas. Two retinas were incubated in 4 ml of medium in tightly capped vials at 25 °C for 1 h with gentle shaking. The gas phase within the vials was O_2/CO_2 (19:1). After the incubation, the tissues were removed, carefully blotted on glass and dropped into liquid N_2 . The frozen tissues were placed in tubes containing 300 μ l of water in a boiling-water bath and dissociated with a Pasteur pipette. The dissociated tissues were incubated at 100 °C for 10 min, after which the suspension was transferred to Microfuge tubes and centrifuged for 5 min at 12000 g. The supernatant was removed and its volume measured.

The pellet was digested in 1 M-NaOH and its protein content determined (Lowry *et al.*, 1951), with bovine serum albumin as the standard.

Rationale for determination of the gluconeogenic pathway

In brief, to determine the activity of the gluconeogenic pathway in liver and kidney, the release of glucose from the tissue incubated in a glucose-free medium is measured or an increase in hepatic glycogen is measured. This particular approach could not be used for the retina, since the retina relies solely on glucose as its energy source (Lolley, 1969; Graymore, 1970) and does not release detectable amounts of glucose to the incubation medium (S. S. Goldman, unpublished work). Therefore it was assumed that retinal glycogen can act as a 'trap' for the glucose derived metabolically from the ^{14}C -labelled non-carbohydrate precursors, as described previously (Goldman & Witkovsky, 1987).

Measurement of the amount of [^{14}C]glucose incorporated into glycogen

Triplicate 100 μl samples of the supernatant were applied to 2 cm $\times$ 2 cm squares of Whatman 31 ET chromatography paper and immersed immediately in 67% (v/v) ethanol. The filter papers were processed as described by Chan & Exton (1976). The dried filter papers were cut into small pieces and incubated in 2 ml of 50 mM-acetate buffer, pH 5.0, containing 50 μg of amyloglucosidase/ml, for 1.5 h at 37 $^{\circ}\text{C}$. After the digestion, a 1 ml sample was removed and counted for ^{14}C radioactivity.

It was shown in previous work, in which retinas were incubated in Ringer containing 2 mM-glutamate and [^3H]glutamate, that a considerable amount of the label incorporated into glycogen was [^3H]glucose (Goldman & Witkovsky, 1987). In the present study it was found that all of the label released from the Whatman 31 ET paper after amyloglucosidase digestion was [^{14}C]glucose. This was done by applying neutralized samples of the amyloglucosidase digest prepared from retinas incubated in either [^{14}C]glutamate or [^{14}C]lactate to AG1-X8 (formate form) anion-exchange resin columns. The material was eluted with water. Over 90% of the radioactivity applied to the column and essentially all of the glucose was recovered. Other neutralized samples were used to convert enzymically the glucose into glucose 6-phosphate by the hexokinase reaction. These fractions were applied to AG1-X8 (formate form) anion-exchange columns, and the material was eluted with water. No radioactivity or glucose was recovered.

The rate of incorporation of glucose into glycogen is expressed in pmol of glucose incorporated/h per mg of protein. The rate of incorporation was corrected for glycogen that sediments with the pellet. Since the ^{14}C skeleton of the uniformly labelled precursors used will be diluted by non-radioactive carbon from other sources within the glycolytic pathway, the specific radioactivity of the newly synthesized glucose will be less than that of the parent compound (Katz, 1985, and references therein). Corrections have not been made for this metabolic dilution of the radioisotope, and the results given here thus may have underestimated the actual rates for gluconeogenesis.

Determination of glycogen

The glycogen content of the retinas was determined as described by Lust *et al.* (1975) on both the supernatant and pellet fractions, since it was found that 25–30% of the glycogen co-sediments with the pellet. The concentration of amyloglucosidase used was increased to 5 μg instead of the 50 ng used by Lust *et al.* (1975). The results are reported as the sum of these fractions.

Determination of lactate in the incubation medium

After the incubation, a sample of the incubation medium was removed and the amount of lactate produced by the tissue was determined enzymically by the procedure of Lowry & Passonneau (1972).

Determination of tissue metabolic intermediates

In another series of experiments, retinas were incubated as described above, but in the absence of radioisotope. After incubation, the tissues were removed and dropped into liquid N_2 . The frozen tissues were immersed into 200 μl of 3 M- HClO_4 (-20°C) and allowed to thaw slowly at -10°C . Neutralized HClO_4 digests of these tissues were prepared as described by Lowry & Passonneau (1972). The metabolic intermediates were determined by fluorimetric assays with a Farrand Ratio 2 System fluorimeter (Farrand Optical Co., Valhalla, NY, U.S.A.). Phosphocreatine and the adenylates were determined as described by Lust *et al.* (1981), and glucose 6-phosphatase, fructose 1,6-bisphosphatase, PEP and pyruvate were determined as described by Lowry & Passonneau (1972).

Assays for the gluconeogenic enzymes

Retinas were homogenized in 400 μl of 50 mM-Tris/HCl, pH 7.4, containing 1 mM-EDTA and 1 mM-dithiothreitol. A sample of the homogenate was removed for protein determination. The crude homogenate was used for all the enzyme assays.

Glucose-6-phosphatase (EC 3.1.3.9). The activity was determined as described by Baginski *et al.* (1974), with the following modifications. A sample of the homogenate was incubated in 50 μl of solution for 15 min at 25 $^{\circ}\text{C}$. After the incubation, the reaction was stopped with 50 μl of 10% (v/v) trichloroacetic acid, and the P_i released was determined as described by Lanzetta *et al.* (1979).

Fructose-1,6-bisphosphatase (EC 3.1.3.11). The activity was determined as described previously (Goldman & Witkovsky, 1987).

Pyruvate carboxylase (EC 6.4.1.1) and PEP carboxykinase (EC 4.1.1.32). The activities of these enzymes were determined by measuring the incorporation of [^{14}C]- HCO_3^- into either pyruvate or PEP. Since the product of these reactions is oxaloacetate, and since oxaloacetate is labile, each of the assay systems contained malate dehydrogenase and NADH to convert the oxaloacetate into malate.

For pyruvate carboxylase, the method was similar to that described by Scrutton *et al.* (1969), except that 150 μg of protein was incubated in 200 μl of solution for 30 min at 25 $^{\circ}\text{C}$. The activity of acetyl-CoA-dependent pyruvate carboxylase is defined as the difference in the activities of the homogenate incubated in the absence and the presence of 0.1 mM-acetyl-CoA.

Table 1. Apparent rates of gluconeogenesis, glycogen content and lactate production of retinas incubated in Ringer containing 50 μ M-glutamate

The conditions for the incubation and the analyses of the tissues are given in the Experimental section. The amphibian Ringer contained 50 μ M-glutamate and 1 μ Ci of [U- 14 C]glutamate/ml. The results are expressed as means $\pm$ S.E.M. The apparent rate of gluconeogenesis is expressed as pmol of glucose incorporated into glycogen/h per mg of protein, the glycogen content of the tissue as nmol of glucosyl units/mg of protein, and lactate production of the tissue as nmol of lactate produced/h per mg of protein. Abbreviations: AOAA, amino-oxyacetic acid; 3-MP, 3-mercaptopycolinic acid. Statistical significances were established by the unpaired two-tailed Student's *t* test; **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

	<i>n</i>	Rate of incorporation	Glycogen content	Lactate production
No additions	10	20.63 $\pm$ 0.83	343 $\pm$ 17	130 $\pm$ 8
100 nM-Glucagon	4	15.25 $\pm$ 1.23**	419 $\pm$ 36*	146 $\pm$ 7
100 nM-VIP	3	28.48 $\pm$ 1.84**	214 $\pm$ 6**	115 $\pm$ 9
2 μ M-A23187	3	34.67 $\pm$ 3.57***	317 $\pm$ 19	214 $\pm$ 8***
55 mM-K ⁺	2	31.55 $\pm$ 1.56***	214 $\pm$ 29**	842 $\pm$ 25***
10 μ M-Monensin	2	25.12 $\pm$ 2.88	246 $\pm$ 11*	1014 $\pm$ 42***
100 μ M-Forskolin + 200 μ M-IBMX	4	21.45 $\pm$ 1.79	272 $\pm$ 41	361 $\pm$ 14***
75 μ M-AOAA	3	10.49 $\pm$ 1.22***	285 $\pm$ 3	366 $\pm$ 14***
500 μ M-AOAA	3	6.09 $\pm$ 0.38***	295 $\pm$ 28	339 $\pm$ 5***
100 μ M-3-MP	3	17.80 $\pm$ 1.08	313 $\pm$ 28	139 $\pm$ 8
750 μ M-3-MP	2	12.95 $\pm$ 0.75**	244 $\pm$ 6*	122 $\pm$ 18

For the PEP carboxykinase reaction the method was similar to that described by Lane *et al.* (1969), except 150 μ g of protein was incubated in 200 μ l of solution for 2 h at 25 °C. The activity of PEP carboxykinase is defined as the difference in the activities of the homogenate incubated in the absence and the presence of 1.25 mM-PEP.

After these incubations, the tubes were removed to a hood, 200 μ l of 10 % trichloroacetic acid was added and the tubes were incubated for 1 h at 37 °C to drive off 14 CO₂. The solution was centrifuged for 5 min at 12000 *g*, and a sample was removed and counted for radioactivity.

Materials

[U- 14 C]Glutamic acid (282 mCi/mmol), sodium [U- 14 C]lactate (179 mCi/mmol), NaH 14 CO₃ (8.4 mCi/mmol), [U- 14 C]glucose (3.7 mCi/mmol) and [3- 3 H]glucose (13.5 Ci/mmol), as well as Aquasol, were purchased from New England Nuclear, Boston, MA, U.S.A. All enzymes used were purchased as freeze-dried powders from Sigma Chemical Co., St. Louis, MO, U.S.A. Ox heart lactate dehydrogenase, ox heart malate dehydrogenase and *Aspergillus niger* amyloglucosidase were purchased as (NH₄)₂SO₄ suspensions. All the biochemicals were purchased from Sigma, except for bovine glucagon, A23187, forskolin and monensin, which were purchased from Calbiochem, La Jolla, CA, U.S.A. The AG-1 X8 (formate form) anion-exchange columns were purchased from Bio-Rad, Richmond, CA, U.S.A. 3-Mercaptopicolinic acid (SK&F 34288) was kindly provided by Smith Kline and French, Philadelphia, PA, U.S.A.

RESULTS

Glutamate as the gluconeogenic precursor

The extracellular concentration of glutamate in amphibian retina may be as low as 2 μ M (Miller *et al.*, 1985). The apparent *K_m* of the high-affinity transport system for glutamate in whole retina is believed to be 15–35 μ M (Neal & White, 1971, 1973; Tunncliffe, 1975). For these

studies 50 μ M-glutamate was chosen. Under these conditions, the rate of incorporation of glucose into glycogen was approx. 21 pmol/h per mg (Table 1). In 1 h the concentration of glutamate in the incubation medium was decreased by approx. 50 % (results not shown). At 100 μ M and 200 μ M-glutamate the rates for the incorporation of glucose into glycogen were 43.0 $\pm$ 5.1 and 48.7 $\pm$ 4.5 (*n* = 4) pmol/h per mg respectively.

Enhanced rates of incorporation were found with the following agents: 2 μ M-A23187, 67 % increase; 55 mM-K⁺, 53 % increase; 100 nM-VIP, 38 % increase; and a modest increase of 22 % with 10 μ M-monensin. On the other hand, a mixture of 100 μ M-forskolin and 200 μ M-IBMX did not alter the rate of incorporation. There was a 26 % decrease with 100 nM-glucagon. The inhibitor for the enzyme aspartate aminotransferase, amino-oxyacetate, caused a 50 % decrease at 75 μ M and a 70 % decrease at 500 μ M. In the presence of 3-mercaptopycolinate, the inhibitor for the enzyme PEP carboxykinase (DiTullio *et al.*, 1974; Jomain-Baum *et al.*, 1976), a 37 % decrease was found at 750 μ M.

The production of lactate as well as the tissue glycogen content were both altered by many of the agents used (Table 1). There was a pronounced increase in lactate production: 6–8-fold by 55 mM-K⁺ and monensin; a 3-fold increase by amino-oxyacetate or by a mixture of forskolin/IBMX; and a small increase, 1.5-fold, by A23187. No increase in lactate production was found for glucagon, VIP or 3-mercaptopycolinate. Decreases in glycogen were found when retinas were incubated in the presence of VIP (37 %), 55 mM-K⁺ or monensin (28–37 %) and 3-mercaptopycolinate (28 %). A small decrease was found in the presence of forskolin/IBMX (20 %). On the other hand, there was a marked increase in the presence of glucagon (22 %).

Lactate as the gluconeogenic precursor

The concentration of lactate used (8–9 mM) is believed to be relevant physiologically in the mammalian retina (Matschinsky *et al.*, 1968; Lolley, 1972; Lolley & Schmidt, 1974). Lactate concentrations in the amphibian

Table 2. Apparent rates of gluconeogenesis, glycogen content and lactate production of retinas incubated in Ringer containing 8.5 mM-lactate

The conditions for the incubation, analyses of the tissue and the expression of the data are similar to those given in Table 1. The amphibian Ringer contained 8.5 mM-sodium lactate and 1 μ Ci of [U- 14 C]lactate/ml. Under all conditions there was no lactate production, i.e. no net increase in lactate concentration.

	<i>n</i>	Rate of incorporation	Glycogen content
No additions	8	1426 $\pm$ 165	305 $\pm$ 18
100 nM-Glucagon	3	754 $\pm$ 24*	389 $\pm$ 4*
100 nM-VIP	3	1179 $\pm$ 109	353 $\pm$ 12
2 μ M-A23187	3	1509 $\pm$ 198	227 $\pm$ 14*
55 mM-K ⁺	3	413 $\pm$ 33**	223 $\pm$ 26*
55 mM-K ⁺ + 0.5 mM-ouabain	3	756 $\pm$ 41*	327 $\pm$ 20
100 μ M-Forskolin + 200 μ M-IBMX	3	221 $\pm$ 10**	291 $\pm$ 22
750 μ M-3-MP	2	852 $\pm$ 91	401 $\pm$ 2*

retina have not been documented. At 8.5 mM-lactate, it was found that the rate of incorporation of glucose into glycogen was increased approx. 70-fold compared with that found for glutamate, 1426 versus 21 pmol/h per mg respectively (Table 2). The rate of incorporation of glucose into glycogen was also dependent on the concentration of lactate: at 1.25 mM-, 2.5 mM-, 5.0 mM- and 10 mM-lactate the rates of incorporation were 273 $\pm$ 38, 747 $\pm$ 45, 1140 $\pm$ 35 and 1556 $\pm$ 110 (*n* = 3) pmol/h per mg respectively.

In the presence of the agents used above, significant decreases in the rates for the incorporation of glucose into glycogen were found with 3-mercaptopycolinate (40%), 100 nM-glucagon (47%), 55 mM-K⁺ (71%), which was reversed partially by 0.5 mM-ouabain and with forskolin/IBMX (85%). Both A23187 and VIP were without effect.

It also was found that incubation of the retina in 8.5 mM-lactate completely suppressed lactate production. The contents of glycogen were increased significantly by glucagon and 3-mercaptopycolinate, by 26 and 32% respectively. A small increase (16%) was found for VIP. On the other hand the glycogen was decreased by 25% by A23187 or 55 mM-K⁺. The decrease by 55 mM-K⁺ was blocked completely by 0.5 mM-ouabain.

Determination of the amount of glucose in glycogen derived through gluconeogenesis

The percentage of glucose incorporated into glycogen that is derived through gluconeogenesis can be estimated with two specifically radiolabelled forms of glucose, [3- 3 H]glucose and [U- 14 C]glucose (Spence & Koudelka, 1985, and references therein). The ratio of the radioactivities of 3 H and 14 C in glycogen relative to their ratio in the incubation medium reflects the amount of glucose derived through gluconeogenesis. Since 3 H is lost at the triose-phosphate isomerase step (Katz *et al.*, 1965), the ratio of the radioactivities of these two isotopes in glycogen will reflect the amount of glucose incorporated into glycogen that was derived from the glycolytic

Table 3. Estimation of the amount of glucose in glycogen derived through gluconeogenesis

The conditions for the incubation and analyses of the tissue are given in the Experimental section. The tissues were incubated in amphibian Ringer containing approx. 1.5 μ Ci of [3- 3 H]glucose/ml and 1.0 μ Ci of [U- 14 C]glucose/ml for 1 h as described. The amounts of 3 H and 14 C both in the incubation medium containing the mixture of the two isotopes and in glycogen were determined in a Beckman LS 3801 liquid-scintillation counter programmed with sealed 3 H and 14 C quenched standards (Amersham, Arlington Heights, IL, U.S.A.). The results are expressed as means $\pm$ S.E.M. (*n* = 4). The ratio of 3 H/ 14 C in the incubation medium is given as means $\pm$ S.E.M. for quadruplicate determinations.

	Expt. I	Expt. II
Medium		
Ratio 3 H/ 14 C	1.430 $\pm$ 0.002	1.582 $\pm$ 0.002
Retinal glycogen		
Ratio 3 H/ 14 C	1.217 $\pm$ 0.015***	1.390 $\pm$ 0.006***
Glucose derived through gluconeogenesis (%)		
Content (nmol/mg of protein)	337 $\pm$ 7	325 $\pm$ 17
Rate of [3 H]glucose incorporation (nmol/h per mg)	8.04 $\pm$ 0.59	11.4 $\pm$ 1.05
Rate of [14 C]glucose incorporation (nmol/h per mg)	9.48 $\pm$ 0.59	12.8 $\pm$ 1.07
Lactate production (nmol/h per mg of protein)	119 $\pm$ 4	114 $\pm$ 8

Table 4. Comparison of the metabolic intermediates of retinas incubated in the absence and the presence of 10 mM-lactate

The conditions for the incubation and analyses of the tissue are given in the Experimental section. The results are expressed as means $\pm$ S.E.M. (*n* = 4). The concentrations of the metabolic intermediates are expressed in nmol/mg of protein, and lactate production in nmol of lactate produced/h per mg of protein; N.P., no lactate production. The energy charge potential is defined as ([ATP] + $\frac{1}{2}$ [ADP])/([ATP] + [ADP] + [AMP]).

	No lactate	With lactate
Energy metabolites		
Phosphocreatine	21.78 $\pm$ 1.63	20.37 $\pm$ 2.78
ATP	19.37 $\pm$ 0.98	20.09 $\pm$ 1.34
ADP	1.99 $\pm$ 0.55	1.35 $\pm$ 0.17
AMP	2.06 $\pm$ 0.56	2.49 $\pm$ 1.28
Energy charge potential	0.869 $\pm$ 0.026	0.874 $\pm$ 0.043
Glycolytic intermediates		
Glucose 6-phosphate	2.07 $\pm$ 0.43	3.40 $\pm$ 0.60
Fructose 1,6-bisphosphate	1.52 $\pm$ 0.13	1.52 $\pm$ 0.09
Phosphoenolpyruvate	2.06 $\pm$ 0.19	1.63 $\pm$ 0.32
Pyruvate	4.17 $\pm$ 0.58	10.24 $\pm$ 0.75*
Lactate production (nmol/h per mg of protein)	123 $\pm$ 6	N.P.

Table 5. Activities of gluconeogenic enzymes in retinal homogenatesResults are means $\pm$ S.E.M. for *n* determinations.

Enzyme	<i>n</i>	Activity nmol/h per mg of protein)
Glucose-6-phosphatase	6	108 $\pm$ 12
Fructose-1,6-bisphosphatase	4	25 $\pm$ 2
Pyruvate carboxylase	4	583 $\pm$ 74
PEP carboxykinase	3	28 $\pm$ 5

intermediates below triose-phosphate isomerase (Spence & Koudelka, 1985). If there is no change in the ratio of these isotopes in glycogen relative to their ratio in the incubation medium, then the glucosyl units in glycogen are derived entirely through the direct pathway. A decrease in this ratio will reflect the amount of glucose metabolized first to the trioses and resynthesized back to glucose. Table 3 summarizes the results of these studies. It was found that in one experiment the ratio of ^3H : ^{14}C in glycogen was 15% less than that for the incubation medium, and in the second experiment the ratio was approx. 11% less.

Amounts of the metabolic intermediates of the retina incubated *in vitro*

The amounts of phosphocreatine, the adenylates and some of the glycolytic intermediates of retinas incubated with and without 10 mM-lactate are summarized in Table 4. Both the amounts of these metabolic intermediates as well as the energy charge potential were essentially the same, except for the 2.5-fold increase in pyruvate in tissues incubated in lactate-containing Ringer.

Activities of the retinal gluconeogenic enzymes

The most active enzyme was the acetyl-CoA-dependent pyruvate carboxylase (Table 5). The activities of PEP carboxykinase and fructose-1,6-bisphosphatase were similar, and were approx. 5% of that of pyruvate carboxylase. The activity of glucose-6-phosphatase was intermediate to that found for the other gluconeogenic enzymes.

DISCUSSION

The gluconeogenic pathway is difficult to quantify accurately in an intact piece of tissue such as the retina that (1) has the highest metabolic rate (Lolley, 1969; Graymore, 1970), (2) relies solely on glucose as its energy source (Lolley, 1969; Graymore, 1970) and (3) does not release detectable amounts of glucose to the incubation medium. The methodology used in the present study defines the gluconeogenic pathway empirically by assuming that retinal glycogen acts as a 'trap' for the glucose derived metabolically from [^{14}C]glutamate or lactate. A number of requirements are inherent to this experimental approach, which does not conform to the standard methodologies used for the classic gluconeogenic tissues. These are: (1) that for the rate of glucose incorporation

into glycogen to reflect the rate of gluconeogenesis, the glycogen contents must remain stable; (2) that the newly synthesized glucose shares or is transported to the metabolic compartment for glycogenesis; and (3) that the newly synthesized glucose is not metabolized.

It has been shown that the turnover of retinal glycogen varies with the degree of illumination (Goldman & Witkovsky, 1986), that the rate of glycogenolysis in retina can be altered by a number of modulators (Schorderet *et al.*, 1984), and that the glycogen contents vary with the pathophysiological state of the retina (Kaskel *et al.*, 1973; Sosula *et al.*, 1974; Mizuno & Sato, 1975). Thus the apparent rate of gluconeogenesis may be affected secondarily by factors that influence glycogen metabolism. In regard to the second assumption, it is not known if the newly synthesized glucose shares all of the cellular compartments for glycogenesis. In amphibian retina, glycogen has been found in many different cell types, which include the paraboloid of certain photoreceptors, Muller cells and other neuronal elements (Yamada, 1960; Schabadash & Schabadash, 1972; Witkovsky & Yang, 1982). However, it must be assumed that the newly synthesized glucose does enter at least one of these compartments, since glycogen is labelled. Finally, it is not known if the newly synthesized glucose is metabolized by the retina. This would be difficult to resolve, since the metabolic intermediates of glucose metabolism are common with those for gluconeogenesis.

In spite of these problems, the evidence for gluconeogenesis is both clear and unequivocal. The amphibian retina does possess a full complement of the enzymes necessary for gluconeogenesis, is able to convert both glutamate and lactate into glucose and can incorporate this newly synthesized glucose into glycogen. Of great interest are the findings that the apparent rate for gluconeogenesis was nearly 70-fold greater with lactate than with glutamate as the precursor and that 11–15% of the glucose units in glycogen was derived from C_3 metabolites (lactate ?) of the glycolytic pathway. This suggests that a considerable amount of the glucose incorporated into glycogen is derived through gluconeogenesis. Further, the gluconeogenic pathway in the amphibian retina may be involved with recycling lactate and not glutamate, as suggested previously (Goldman & Witkovsky, 1987). It is conceivable that lactate produced by certain cells in the retina is converted back into glucose, stored in glycogen and used as a carbon source to support the metabolism of other retinal neurons. In order to recycle lactate, there must be a reversible lactate dehydrogenase isoenzyme. Retinal lactate dehydrogenase activity is high (Lolley, 1969; Graymore, 1970), and histochemically the enzyme has been localized in large part to the Muller (glial) cell (Cogan & Kuwabara, 1959; Kuwabara & Cogan, 1959). Histochemically, the lactate dehydrogenase reaction must be driven in the direction of pyruvate (Pearse, 1972), which may suggest that the Muller cell contains this particular lactate dehydrogenase isoenzyme. On the basis of distribution of phosphorylase activity, the Muller cell in the amphibian retina may account for 15–30% of retinal glycogen (Dick, 1984). It is conceivable that most of the glycogen in the Muller cell is derived through the gluconeogenic pathway. If this is the case, presuming our original supposition that the Muller cell provisions certain neighbouring neurons with glucose, then the glucose derived through the gluconeogenic pathway (Goldman & Witkovsky, 1987),

may be especially important with respect to its presumed supportive role in the retina.

Characterization of the gluconeogenic pathway

The strategy used to characterize the properties of the retinal gluconeogenic pathway was to assume that there may be some similarity to the mammalian liver with respect to the extra- and intra-cellular signals that modulate the activity of the pathway. The intracellular signals that stimulate hepatic gluconeogenesis as well as the turnover of hepatic glycogen are believed to be increases in cytosolic cyclic AMP and Ca^{2+} (Exton, 1982; Kraus-Friedmann, 1984).

It was found that glucagon, the classic gluconeogenic and glycogenolytic hormone, not only inhibited the incorporation of glucose into glycogen, but it also produced an increase in tissue glycogen. These observations differ from those observed for mammalian hepatic tissue (Exton, 1981). If this unique glucagon effect is dependent on a cyclic AMP-dependent mechanism, then incubation of the tissue in the presence of forskolin/IBMX should mimic in part this glucagon effect. In lactate-containing Ringer, there was a profound decrease in the rate of gluconeogenesis, whereas in glutamate-containing Ringer no effect on gluconeogenesis was seen. It has been reported that glucagon is not an effective agent for increasing cyclic AMP in retina or brain, and is not glycogenolytic in brain (Watling & Dowling, 1983; Magistretti *et al.*, 1981). It would appear that in the amphibian retina glucagon possesses both anti-gluconeogenic and glycogenic properties. On the other hand, VIP is glycogenolytic in both brain and retina (Magistretti *et al.*, 1981; Schorderet *et al.*, 1984) and is believed to involve a cyclic AMP-dependent pathway (Magistretti *et al.*, 1981; Magistretti & Schorderet, 1984). In the absence of lactate, VIP possessed both gluconeogenic and glycogenolytic activity, whereas in the presence of lactate, VIP, although less potent, acted similarly to glucagon. These results suggest that the presence of lactate confers on the tissue a different physiological state in which the cyclic-AMP-dependent pathways appear to be altered.

In order to evaluate the effects of increased cytosolic Ca^{2+} , the Ca^{2+} ionophore A23187 was used. In glutamate-containing Ringer, there was an enhanced rate of gluconeogenesis, whereas the ionophore was ineffective in lactate-containing Ringer.

Effects of inhibitors on the gluconeogenic pathway

The effect of the inhibitor 3-mercaptopycolinate demonstrates that the conversion of both glutamate and lactate into glucose is dependent on PEP carboxykinase (DiTullio *et al.*, 1974; Jomain-Baum *et al.*, 1976), which has been demonstrated in the retina (Table 5). However, the inhibitor was less effective in amphibian than in mammalian tissue (DiTullio *et al.*, 1974; Jomain-Baum *et al.*, 1976). This may reflect species and/or tissue differences with respect to the kinetic properties of the enzyme.

Further, it was shown that amino-oxyacetate was a potent inhibitor when glutamate was the precursor, suggesting that entry of glutamate into the citric acid cycle may occur predominantly through the aspartate transaminase reaction. Higher concentrations of amino-oxyacetate were avoided, since this compound reacts readily with 2-oxo acids (Meijer & Van Dam, 1974), and

elevated concentrations of the inhibitor might have compromised metabolism. Therefore the participation of glutamate dehydrogenase cannot be ruled out as a second entry pathway into the citric acid cycle.

Relationship between gluconeogenesis and energy metabolism

Since the retina produces prodigious amounts of lactate aerobically (Lolley, 1969; Graymore, 1970), the rate of its production has been used to monitor energy metabolism (Winkler, 1981). However, the high aerobic glycolytic capacity of the retina has been observed only *in vitro* (Lolley, 1969; Graymore, 1970) and its capacity *in vivo* still remains uncertain (Tornquist & Alm, 1979). In the presence of millimolar concentrations of lactate, which mimic the conditions *in vivo* (Matschinsky *et al.*, 1968; Lolley, 1972; Lolley & Schmidt, 1974), lactate production is extinguished completely, or at least is suppressed to undetectable values (Tables 2 and 4). This suppression of lactate production does not reflect an impairment in metabolism, since the metabolic integrity of the tissue was not altered (Table 4). This unique metabolic property of the amphibian retina also is shared by the mammalian retina (Lolley, 1972; Lolley & Schmidt, 1974) and, as suggested by those investigators, this property may reflect the actual metabolic status of the tissue. Therefore a second indicator for measuring retinal energy metabolism must be used.

The steady-state content of glycogen in nervous tissue reflects the balance between the rates of glycogenesis and glycogenolysis, and further, an alteration in these steady-state contents is believed to reflect the degree of metabolic activity (Seijo, 1978). The decrease in tissue glycogen by 55 mM- K^+ and its normalization in the presence of ouabain may be relevant physiologically. It suggests that the increased glycogenolytic effect by elevated $[\text{K}^+]$ is linked metabolically to the Na^+ pump, probably reflecting the greater demand for glucose by the tissue to support the increased ATP requirements of the pump (Mata *et al.*, 1980; Siegal *et al.*, 1981). Since gluconeogenesis is an ATP-demanding process (Lehninger, 1977), the reciprocal effect of elevated $[\text{K}^+]$ and ouabain on the rate of gluconeogenesis may reflect competition for ATP between the Na^+ pump and gluconeogenesis. Since the metabolic intermediates of the glycolytic and gluconeogenic pathways are common, the rate of flux of these metabolites through these two opposing pathways must be controlled. It was shown that fructose 2,6-bisphosphate inhibits retinal fructose-1,6-bisphosphatase (Goldman & Witkovsky, 1987), suggesting that this enzyme also may possess similar control mechanisms to that reported for the mammalian enzyme (Van Schaftingen & Hers, 1981; Pilkis *et al.*, 1981). This particular control point may be important with respect to regulating the flux of glucose between glycolysis and gluconeogenesis (Claus *et al.*, 1984; Hue & Rider, 1987).

These studies demonstrate, as well as broaden our understanding of, the complexities of both retinal energy metabolism and gluconeogenesis. They also encourage further investigations and emphasize the importance of lactate.

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